Proteomics a leading technology for Development of Novel Cancer Biomarker

Desh Deepak Singh

Department of Microbiology, Chhatrapati Shahuji Maharaj Medical University, Chowk, Lucknow U.P. INDIA- 226003
ddsbms@gmail.com

ABSTRACT:

A handful of cancer biomarkers are currently used routinely for population screening, disease diagnosis, prognosis, monitoring of therapy, and prediction of therapeutic response. The emergence of novel technologies allows researchers to facilitate the comprehensive analyses of genomes, transcriptome, and proteomes in health and disease. The information that is expected from such technologies may soon exert a dramatic change in the pace of cancer research and impact dramatically on the care of cancer patients. These approaches have already demonstrated the power of molecular medicine in discriminating among disease subtypes that are not recognizable by traditional pathologic criteria and in identifying specific genetic events involved in cancer progression. This review covers a selection of advances in the empire of proteomics and its promise for cancer biomarker discovery. It also addresses issues regarding sample preparation and specificity and discusses current challenges that need to be overcome. Finally, the review touches on the efforts of the Early Detection Research Network at the National Cancer Institute in promoting biomarker discovery for translation at the clinical level.

Key words: Proteomics, Cancer, biomarker discovery, Differential Gel Electrophoresis, Multidimensional Chromatography, Bioinformatics, Protein identification.

INTRODUCTION

Over 11 million people are diagnosing with cancer each year. It is predictable that in present will be 16 million novel cases each year next to 2020. Commencing a total of 58 million deaths international in 2005, cancer accounts for 7.6 million (or 13%) of the global mortality [1]. Deaths from cancer in the humankind are anticipated to keep on rising, among a predictable 9 million community disappearing from cancer in 2015 and 11.4 million dying in 2030. Since a significant natural indicator of cancer position and development for the physiological state of the cell at a precise time, biomarkers correspond to powerful tools for supervise the course of cancer and gauging the efficiency and security of original beneficial agent [2]. They can have wonderful healing impact in diagnostics oncology, particularly if the biomarker is detected before clinical indication or enable real-time supervising of drug response. There is a critical need for speed up development of biomarkers and their use to get better diagnosis for cancer. Malignant Transformation engages modifications in protein expression with successive cloned production of the changed cells. These alterations can be monitored at the protein level, both qualitatively and quantitatively [3]. Protein signatures in cancer provide valuable information that may be an aid to more effective diagnosis, prognosis, and response to therapy. Oncoproteomics is learning of proteins and their interactions in a cancer cell by proteomic technologies. Proteomic research first came to the fore with the introduction of two-dimensional gel electrophoresis. At the turn of the century, proteomics has been increasingly applied to cancer research with the wide-spread introduction of mass spectrometry and protein chip [4]. There is an intense interest in applying proteomics to foster an improved understanding of cancer pathogenesis, develop new tumor biomarkers for diagnosis, and early detection using proteomic Portrait of samples [5]. Oncoproteomics has the potential to revolutionize clinical practice, including Cancer diagnosis and screening based on proteomic platforms as a complement to histopathology, individualized selection of therapeutic combinations that target the entire cancer-specific protein network, real-time assessment of therapeutic efficacy and toxicity, and rational modulation of therapy based on changes in the cancer protein network associated with prognosis and drug resistance [6]. Besides, oncoproteomics is also applied to the discovery of new therapeutic targets and to the study of drug effects.

In pace with the successful completion of the Human Genome Project, the wave of proteomics has raised the curtain on the post genome era. The study of oncoproteomics provides mankind with a better understanding of neoplasia [7]. In this article, the discovery of cancer biomarkers in recent years is reviewed. The challenges ahead and perspectives of oncoproteomics for biomarkers development are also addressed. With a wealth of information that can be applied to a broad spectrum of biomarker research projects, this review serves as a reference for biomarker researchers, scientists working in proteomics and bioinformatics, oncologists, pharmaceutical scientists, biochemists, biologists, and chemists [8].

Genomics-based approaches to biomarker development include the measurements of expression of full sets of mRNA, such as differential display serial analysis of gene expression and large-scale gene expression arrays. However, interpreting the best data and adapting the results to a particular application remain challenging. Although studies of differential mRNA expression are informative, they do not always correlate with protein concentrations [9,10] . Proteins are often subject to proteolytic cleavage or posttranslational modifications, such as phosphorylation or glycosylation.

Cancer biomarker discovery strategies that target expressed proteins are becoming increasingly popular because proteomic approaches characterize the proteins, modified or unmodified, involved in cancer progression [11]. Two-dimensional gel electrophoresis has been the mainstay of electrophoretic technology for a decade and is the most widely used tool for separating proteins. Initially described 25 years ago , proteins in a two-dimensional gel are separated in the first dimension based on their isoelectric points and then in a second dimension based on their molecular masses [12].

In many cases, two-dimensional gel electrophoresis may evaluate whole-cell or tissue protein extracts. The use of narrow immobilized pH gradients for the first dimension increases resolving power and can help detect low-abundance proteins [13]. Radioactive or fluorescent labeling and silver staining allow visualization of hundreds of proteins in a single gel. Differences between the samples can be compared and relative quantities determined by quantifying the ratios of spot intensities in independent two-dimensional gels. Matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)4 allows the analysis and identification of very small amounts of protein isolated from the gel *(14–18)*. These advances have combined to make two-dimensional electrophoresis a more attractive option for the analysis of complex protein mixtures. A brief overview of complementary and other rapidly evolving proteomic technologies is provided below.

SAMPLE SEPARATION

For the reason that of its difficulty the serum or plasma proteome cannot be determined entirely via a single proteomic equipment. Manifold proteomics techniques for protein severance must be collective to examine and cover up a huge range of the proteome (Figure 1). Which methods are desired depends on the fundamental natal and medical questions to be answered. Core technologies for protein separation are one- and two-dimensional gel electrophoresis (1-DE, 2-DE) and, for protein or peptide separation, one- and

two dimensional liquid chromatography (1DLC, 2DLC), all coupled with mass spectrometry (MS) [14].

MULTIDIMENTIONAL CHROMATOGRAPHY

Recently, other methods for separating proteins have been exploited in proteomic analysis. Many involve liquid chromatography technologies that use solid- and liquid-phase media to separate proteins and/or their peptide fragments. The basic principle is a soluble sample that is separated in a liquid-phase through a column, which is usually a tube packed with small particles of specific surface chemistry [15]. The sample is resolved as it traverses the length of the column based on protein- or peptide-specific chemical or physical interactions with the solid-phase [16]. The time when the separated sample is detected at the end of the column (e.g. by UV absorbance at 210 nm, which essentially measures the number and quantity of peptide bonds) is the retention time and is quantitative if the peak contains a single protein/peptide (which in proteomics is rare and therefore, peak volume or intensity in this case is semi-quantitative) [17]. One-dimensional liquid chromatography (1DLC) can be used to separate proteins according to their molecular mass, isoelectric point, or hydrophobicity, which are the three chemical characteristics that define any given protein [18]. The most commonly used 1DLC is reversed phase chromatography, in which proteins are separated based on hydrophobicity [19]. Reversed phase chromatography can also be used to concentrate and/or desalt samples. In 2DLC, proteins are separated in the first dimension by chromatographic focusing (p*I*) and in the Second dimension by reversed phase chromatography (hydrophobicity) [20]. Thus, 2DLC increases the extent of protein fractionation, which facilitates analysis of a larger spectrum of the proteome, including specific isoforms, PTMs and low-abundance proteins [21]. As with 1DLC, this method has been used in proteomics primarily for peptide separation before MS analysis (due to its compatibility with ESI instruments); however it is increasingly used to separate complex intact protein mixtures, which are then enzymatically digested for LC or MALDI (matrix-assisted laser desorption/ionization) MS/MS analysis [22]. 2DLC requires a larger quantity of sample for a single run (>2.5 ml) as compared to 1DLC (50-100 μl), which can be a difficulty if available sample volumes are small (e.g. from mouse models) [23]. It is important both to quantify and to identify proteins present in fractions generated by 1DLC or 2DLC [24]. One strategy is to normalize, overlay and compare elution profiles between different samples using specialized software packages (for which there is currently a need especially when analyzing a large number of samples) and analyze, using MS, only the fraction that varies between samples [25].

Current data suggests that using multiple proteomic technologies dramatically increases the number of proteins detected, especially of those present in the sample at very low abundance [26]. 2-DE, 1DLC and 2DLC are synergistic separation techniques that, coupled with MS identification, expand the observable proteome and will provide a large dynamic protein spectrum for biomarker discovery [25-30]. In fact, we recently compared 2-DE and 2DLC by creating a large database for serum and isolated inner mitochondrial subproteome, and revealed that only about 12% of identified proteins were common to both platforms [31-33]

DIFFERENTIAL GEL ELECTROPHORESIS

Differential Gel Electrophoresis (DIGE) is designed to provide a quantitative component to proteomics experiments utilizing two-dimensional (2D) gel electrophoresis [38]. DIGE can provide detection of changes in protein abundance (sometimes subtle) with statistical confidence while controlling for gel-to-gel variation and other variations of non-biological origin [39]. Advantage of DIGE is the labeled samples are mix and then separated on the same 2-D PAGE gel [40]. Consequently, for samples on the same DiGE gel, gel-to-gel variation is completely eliminated, and the number of gels needed for one experiment can be cut two- to three-fold. One of the three samples on a DiGE gel can be a mixture of equal amounts of all experimental samples, a "pooled internal standard" [41].This creates a standard for each protein in the analysis. Therefore, comparisons across different gels can be made with a high degree of assurance large format gels are cumbersome to handle [42]. Since in DIGE the proteins are pre-labeled, DIGE gels do not have to be manipulated after electrophoresis. Additionally, the scanner that is used for imaging accepts gel sandwiches including the glass plates [43]. This further reduces the variation between gels, and the risk of damaging or destroying gels [44].

Consider a simple experiment with 6 samples commencement the group Disease and 6 samples from the group Control. These 12 samples will be run in 6 gels, with two samples per gel, using Cy3 and Cy5 dyes [45] .One opportunity could be to run the samples from the Disease group in three of the gels, and to run the samples from the Control group in the remaining three gels [46]. Nevertheless, doing so would introduce an artificially great degree of similarity *within* the groups, because variation will be lower between samples in the same gel compared to samples in different gels [47]. A better option is to run one sample from each condition in each of the gels, so that every gel contains one sample from the Disease group and another sample from the Control group, alternating between dyes [48]. This option may appear more valid than the first but in fact it suffers from the opposite problem there will exist an artificially great

similarity *between* conditions because samples from different groups are run in the same gel. As a result, the t-tests will result in a smaller number of significant results [49].

Samples diseased and control are differentially labeled in the midst of spectrally resolvable fluorescent dyes; Cy2, Cy3 and Cy5 and co-resolved on a solitary 2D gel for straight quantitation [50]. By means of internal standards and investigational replication, particular and multi-modification analyses are able to be inter-compared with a comparatively little figure of synchronized DIGE gels [51]. 2-D DIGE technology is based on the precise properties of spectrally resolvable dyes, the CyDye DIGE Fluors [52]. Two sets of dyes are accessible—Cy2, Cy3, and Cy5 minimal dyes, and Cy3 and Cy5 diffusion dyes so as to have been designed to be both mass and charge coordinated [53]. Consequently, indistinguishable proteins labeled with each of the CyDye DIGE Fluors determination shift approximately to the same position on a 2-D gel [54].CyDyes propose immense compassion, detecting as modest as 125 pg of protein and charitable a linear response to protein awareness of up and regarding to four instructions of importance [55] During assessment silver staining detects 1–60 ng of protein by means of less than a hundred-fold lively variety [56]. Labeling does not hamper through succeeding recognition by mass spectrometry (MS) of proteins excised from 2-D DIGE gels, since a good number peptides will not contain a label [57]. Nevertheless, together the mass and hydrophobicity of the CyDyes manipulate protein immigration for the duration of the second dimension of electrophoresis, SDS-PAGE [58]. Because a consequence, modestly labeled 2-D DIGE gels are frequently post-stained, the majority established 2-D DIGE procedure uses N-hydroxysuccinimide ester reagents for low-stoichiometry labeling of amino groups of lysine side chains labeling reactions are standardized so that only 2–4% of the lysine residues are labeled [59]. During minimal labeling, the interior standard is characteristically labeled with Cy2, at the same time as samples are labeled with Cy3 and Cy5 [60]. This course of action uses maleimide reagents for labeling all cysteine sulfhydryls in a sample [61] .It is measured meant for use in situation wherever sample profusion is limited [62] Diffusion labeling is to a great extent more sensitive than minimal labeling, as additional fluorophore is included into each protein species [63]. During diffusion labeling, where a Cy2 fluor is not available, the interior typical is labeled with one of the CyDyes and samples are labeled with the other CyDye [64]. Suspicious optimization of diffusion labeling situation is appreciative to survive accepted out for every new sample set to make sure absolute decline and stiochiometric labeling of cysteine residues [65]. In addition, proteins so as to do

not have cysteine will not be labeled and determination consequently not be imaged [66]

TWO DIMENSIONAL GEL ELECTROPHORESIS

The first technology to be used in proteomics was 2-DE, which was developed independently in the laboratories of O`Farrell and Klose more than three decades ago [67-69]. In standard 2- DE, proteins are separated in the first dimension, known as isoelectric focusing, by their molecular charge (pI). The second dimension separates the proteins according to their molecular mass (or molecular weight, MW). The MW separation is done in a polyacrylamide matrix in a sodium dodecylsulfate (SDS) milieu; the most common procedure utilizes an acrylamide gradient of 10 to 20%. Proteins can be visualized in 2-D gels using different detection methods [70]. The more common protein staining methods include Coomassie blue and silver staining, use of fluorescence dye (e.g. Cy dyes, LAVAPurple, Sypro dyes), radiolabeling, and immunodetection. Using standard format SDS-gels for 2-DE, it is possible to routinely separate up to 2000 protein spots from serum/plasma or tissue extracts, which reflects ~100–300 different proteins, depending on the pH gradient used in the first dimension [71,72]. Although 2-DE is an important and popular protein separation technique, it is limited by the solubility and mass of the separated proteins. Differential in-gel electrophoresis (2D-DIGE) is a recent improvement of the 2-DE technology. It improves gel reproducibility, minimizes alignment issues and allows better quantitative comparison between samples. In 2D-DIGE, proteins from different disease states are separately labeled with different fluorescent dyes, and an internal pooled standard is labeled with another dye [73] . The labeled samples are then combined and subjected to 2-DE, and the gel is scanned at different emission wavelengths generating multiple images that can be overlaid. Figure 4 shows an example of a 2D-DIGE, which allows the differentially regulated proteins to be viewed as changed in color. 2-D gel images are evaluated and analyzed using specialized software packages [74].

The software stores all of the relevant information on each and all of the spots of a 2-D gel in a database, compares gel patterns using complex algorithms, and highlights differences between gel images. 2-D image analysis can be time-consuming and difficult, particularly if there are marked differences between samples. Software packages can be purchased and used in-house for analysis, or companies will now provide image analysis on a contract basis. However, by using strict inclusion and exclusion criteria one can sieve out the high probability markers (or protein spot changes).

PROTEIN IDENTIFICATION

Bioinformatics is a branch of in sequence knowledge regarding the luggage compartment space, salvage, apparition, calculation and investigation of molecular data with biological consequence. Bioinformatics can go faster proteomic studies in data pulling out, incorporated data supervision and association modeling [75-79]. Data taking out is a process which is at the present renowned as an involvement device in proteomics. This is suitable to the expansion of a spacious variety of software programs enthusiastic to withdrawal data obtained by the side of dissimilar stages of proteomic study. MS results are capable to be compared through sets of hypothetical protein sequences accessible in databases [80-82]. Incorporated data used to put together data acquired beginning proteomics of a variety of areas of interest inside a software atmosphere in regulate to advance the consistency and to allocate improved thoughtful of results [77]. Arrangement modeling and systems biology make available in sequence intended for enhanced thoughtful of huge molecular networks in their cellular situation by *in silico* modeling of the intricacy of biological processes with reference interacting molecules . In drug discovery channel, solitary of the preponderance significant stepladder is the resolve of three-dimensional arrangement of a target protein or nucleic acid [83-90] Bioinformatics software can use the three-dimensional structural in sequence of the unleaded objective to design entirely new lead compounds *de novo*.

Given the low abundance in serum and plasma of known cancer biomarkers, the issue is whether current proteomic technologies provide sufficient depth of analysis for biomarker discovery. Several recent studies have investigated the extent to which proteomic technologies can unravel the complexity of the proteome [90-105]. Diverse methods and instruments were used to compare and integrate tandem mass spectrometry (MS/MS) data from aliquots of pooled serum and plasma from healthy subjects. The concentrations of some of the identified proteins are well within the range of known cancer biomarkers, and the list of high-confidence protein identifications includes proteins such as CA125, which clearly points to the ability to identify low-abundance proteins. Another study tested the assumption that proteins derived from a variety of tissues could be detected in plasma by using current proteomic technologies [105-108]. Results obtained indicate that such tissue-derived proteins were detectable in plasma by direct mass-spectrometric analysis of captured glycopeptides, thus providing support for plasma-based protein biomarker discovery. Further evidence that substantial depth of analysis is currently achievable with fractionation comes from a plasma study following a two-dimensional protein-fractionation schema that resulted

in high-confidence identification of 1,662 proteins and included protein isoforms that differed in their chromatographic mobility. Interestingly, further increase in depth of analysis was accomplished simply by repeat analysis of aliquots from the same set of plasma fractions [109]. Repeat analysis overcomes partial sampling of low-abundance peptides in single liquid chromatography– MS/MS analyses. Thus, current schemes for analysis of a plasma protein subset (for example, glycoproteins or fractionated plasma) allow sufficient depth of analysis to identify circulating cancer biomarkers [110]. Apart from the technological challenge presented by the range of protein concentrations, the profiling of serum or plasma for cancer biomarker identification is difficult in numerous other ways. Perhaps the most important are the sources of variability that contribute to a false discovery and therefore have to be taken into consideration as part of the experimental design and data interpretation [111]. Artefacts or nonspecific disease-associated changes in plasma proteins need to be distinguished from potentially specific biomarkers [112]. This is highly pertinent because cancer-serum protein profiling by MALDI mass spectrometry has uncovered mass profiles for previously unidentified proteins that were proposed to be diagnostic for several common types of cancer [113]. Therefore, the likely correspondence of the mass peaks to relatively abundant serum proteins, findings suggest that profiling of relatively abundant serum proteins may yield distinct differences between cancers and controls. However, such dif ferences may not be specific for cancer or a cancer type, and their utility as part of a strategy for detecting cancer has yet to be Determined [114,115].

MALDI
(Matrix-assisted laser desorption/ionization)
This method requires substantial amounts of materials, and its low sensitivity makes it difficult to sequence those regulatory proteins involved in cancer progression, which are often present in low abundance [116]. Therefore, biochemical studies of cancer biology require more sensitive tools. MALDI has helped to establish the MS platform as an important tool in proteomics [117]. This technique of ionization has been instrumental in bringing the mass spectrometer, which measures the mass of a molecule, to the forefront of proteomics research [118]. MALDI enables conversion of biomolecules into a charged gaseous state that is essential for analysis by the mass spectrometer. Developed by Karas and Hillenkamp , the procedure involves the precipitation of the sample molecules with an excess of matrix material, such as 3-cyano -4- hydroxycinnamic acid or dihydroxybenzoic acid [119].The precipitated solid is then irradiated with laser pulses, and the matrix material imparts energy to the biomolecules. The matrix materials have absorbances at the wavelength of the laser, and the

molecules are subjected to a process of desorption and ionization accompanied by fragmentation [120]. The mass spectrometer then measures the mass-tocharge ratio (*m/z*) of the protein, peptide, or peptide fragments. Mass separation can be achieved based on the TOF, generation of quadrupole electric fields, or ejection of ions from an ion trap. MALDI can be linked to any one of the above three methods. In the MALDI-TOF MS platform, irradiation by laser pulses produces short bursts of ions that are then accelerated through a flight tube, the smaller ions possessing a higher velocity relative to the larger ions [121]. The velocities are detected, creating a TOF spectrum. The upstream elements of protein purification have the most impact on the output from an MS platform. Analysis is usually carried out after enzymatic degradation of gel-separated proteins. In other instances, intact proteins are digested and analyzed as complex peptide mixtures, without electrophoresis. The molecular masses of peptides derived from the digestion of a specific target represent the fingerprint or protein profile. With an appropriate protein sequence database and a search program (PepFrag or ProFound; available at http://prowl.rockefeller.edu/), mass information can be used for protein identification [122]. Alternatively, the masses of peptide fragments can be compared with those of theoretical peptide fragments specified by the parent peptide mass.
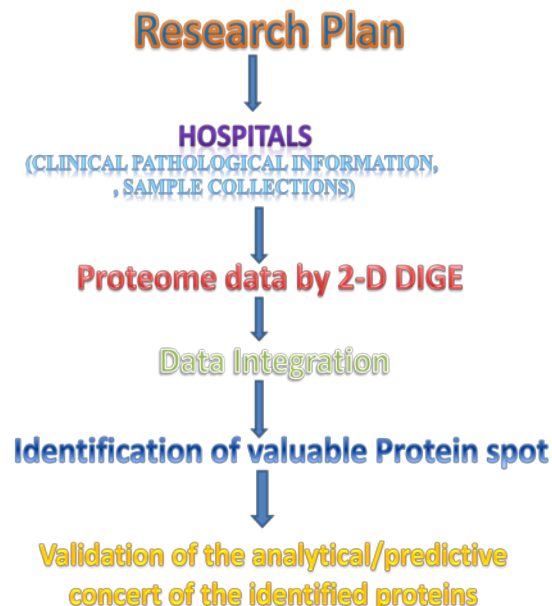
Fig: 1. — Summary diagram demonstrate the Development of biomarkers

MASS SPECTROMETRY
Mass spectrometry techniques have greatly advanced proteomics and proteomics-based biomarker discovery in recent years. 2-DE coupled with MS is widely used for tissue and serum analyses [123-125]. Spots from 2-

D gels are excised, the proteins subjected to in-gel digestion and the resulting peptide fragments identified by MS [129-132]. For identification of 2-D gel spots matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) MS is commonly used [126-128]. If information on protein isoforms, PTMs or "absolute" identification of proteins in complex mixtures such as fractions generated by 1DLC and 2DLC are needed, then tandem MS (commonly referred as MS/MS) is required [129-132]. MS/MS spectra are usually generated by an ion trap or quadrupole time-of-flight mass spectrometer, which allows generating *de novo* sequencing and exact localization of PTMs. For protein quantitation in MS analysis, several isotopic labeling techniques (e.g. iTRAQ, 16O/18O, SILCA) and, recently, label-free methods have been developed [133-135].

Validation for Biomarker

Biomarker candidates have traditionally been evaluated with quantitative immunoassays (e.g. ELISA) that are unique for one analyte [136]. With the rapid development of new potential biomarkers, it is important to develop quantitative assay platforms that can simultaneously measure many proteins in many samples at a small sample volume [137]. A variety of multiplex immunoassays have been developed in recent years, which offer some advantages over traditional quantitative assays [138]. Multiplex immunoassays are essentially the same as an ELISA except that multiple analytes are quantified simultaneously [139]. Thus, many biomarkers can be evaluated at one time under the same standardized conditions, quantitative information can be obtained in a highly parallel analysis, and reagent costs are substantially reduced[140]. The most common multiplex assay used is an array of antibodies printed on slides/or plates at high density [141]. It is now possible to print hundreds of antibodies, although issues with analyte and antibody cross reactivity and matrix affects make smaller numbers (<20) the preferred choice of many [142]. The current issues with multiplex arrays are their inter- and intra-assay reproducibility, matrix affects, background limits and the specificity and sensitivity of the antibody assay [143]. There are many other quantitative and semiquantitative multiplex immunoassays, such as miniature sandwich immunoassays, bead-based multiplex immunoassays and assays for specific signaling pathways, but investigators must take care to ensure the specificity and reproducibility of each assay within the multiplex [144-146]. The ultimate success of a multiplex assay depends upon its ability to quantitatively detect proteins at concentrations likely to be present in serum samples, which range from less than 1 pg/ml to over 1 mg/ml [147]. Multiplex assays can be used as powerful validation tool for candidate biomarkers identified by a "de nova" proteomic

discovery approach. In addition, multiplex assays are often used for evaluating a variety of candidate biomarkers in a "targeted" approach [148]. In either case, the multiplex assay requires the added flexibility of allowing the investigator to mount their own analytes. To test whether or not a newly discovered biomarker is of clinical utility, we recommend evaluating all candidates in relation to existing biomarkers if such exist. Multiplex immunoassays again are a desirable platform for this approach as it provides quantitative information in a higher-throughput format [149]. Biomarkers can be correlated with biological events during drug development in order to validate drug targets or to predict drug response; biomarkers can be used as companion diagnostics in drug development to characterize patient populations in order to better understand the extent to which new drugs reach intended therapeutic targets can alter proposed therapeutic pathways and achieve successful clinical[150].outcomes; biomarkers can be used to stratify patient populations for drug response in primary prevention or disease-modification studies, particularly in specific clinical areas such as neuron degeneration and cancer; clinically useful biomarkers are becoming increasingly useful to make proper therapeutic decisions regarding candidate drugs; and clinically useful biomarkers are becoming increasingly required and other outside authorities to make proper regulatory decisions regarding candidate drugs [151,152, 153].

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